in vivo *34*: 461-467 (2020)
doi:10.21873/invivo.11796

Impact of the *ESM-1* Gene Expression on Outcomes in Stage II/III Gastric Cancer Patients Who Received Adjuvant S-1 Chemotherapy

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Abstract. *Background/Aim: Endothelial cell-specific molecule-1 (ESM-1) is a soluble proteoglycan which has important role in various biological events. We investigated the impact of the ESM-1 expression in cancer tissues on outcomes in stage II/III gastric cancer patients who received adjuvant S-1 chemotherapy. Patients and Methods: The ESM-1 mRNA expression in cancerous tissues and adjacent normal mucosa from 253 patients was measured. The associations between the ESM-1 gene expression and the survival and clinicopathological features were investigated. Results: A significant association was observed between high ESM-1 expression and undifferentiated adenocarcinoma. The overall survival curve was significantly lower in patients with high ESM-1 expression than in those with low expression (p=0.005). High ESM-1 expression was a significant independent prognosticator (HR=2.291, p=0.007). Conclusion: ESM-1 gene expression in cancerous tissues is an important prognosticator in stage II/III gastric cancer patients who received adjuvant S-1 chemotherapy.*

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Key Words: Gastric cancer, endothelial cell-specific molecule-1, ESM-1, adjuvant chemotherapy, S-1.

Gastric cancer is the fifth most major cancer among new cases and the third most common cause of cancer-associated mortality worldwide, numbering 1,033,701 and 781,631 in 2018, respectively (1). The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) demonstrated that adjuvant S-1 chemotherapy significantly improved the survival of patients who received D2 gastrectomy for pathological stage (pStage) II/III gastric cancer (2, 3). Furthermore, in the CLASSIC trial and JACCRO-07 trial, the effectiveness of capecitabine plus oxaliplatin therapy for pStage II/III gastric cancer and S-1 plus docetaxel therapy for pStage III was confirmed (4-6). Thus, fluoropyrimidine remains the key drug for adjuvant chemotherapy in gastric cancer patients (7).

Endothelial cell-specific molecule-1 (ESM-1) was originally cloned from a human endothelial cell cDNA

Table I. *The PCR primer sequences of ESM-1 and β-actin.*

Primer	Sequence
ESM-1	
Sense primer	5'-AAGGCTGCTGATGTAGTTC-3'
Antisense primer	5'-GCTATTTATGGAAGTGTATGTGTTT-3'
β-actin	
Sense primer	5'-AGTCAGCCGCAT CTTCTT-3'
Antisense primer	5'-GCCCAATACGACCAAATCC-3'

library in 1996 (8). ESM1 is a soluble dermatan proteoglycan that can circulate in the bloodstream (9). The structure of ESM-1 is composed of a mature polypeptide of 165 amino acids, of which approximately 30 kDa corresponds to a single dermatan sulfate chain (8). Recently, several authors have shown that ESM-1 is overexpressed at the mRNA and protein levels in various cancers (10-15). It was reported that the overexpression of ESM-1 plays an important role in development, angiogenesis, tumor growth, and so on (16). Furthermore, previous studies have reported that ESM-1 overexpression in tumor tissue was related to poor outcomes in patients with various malignancies (17-20). However, there is no available information concerning the correlation between the ESM-1 expression and long-term outcome in gastric cancer patients who receive adjuvant S-1 chemotherapy.

Thus, we examined the impact of *ESM-1* mRNA expression in cancer tissues regarding outcomes in patients treated with adjuvant S-1 chemotherapy for pStage II/III gastric cancer.

Patients and Methods

Patients and samples. We retrospectively analyzed the clinical data from 146 consecutive patients who underwent curative resection followed by adjuvant S-1 chemotherapy for the treatment of pStage II/III gastric cancer at Kanagawa Cancer Center and Yokohama City University between 2002 and 2010. As a comparison group, we concurrently studied the ESM-1 expression and long-term outcome in 107 patients who did not receive adjuvant chemotherapy with S-1.

Each tissue sample was engrafted in optimum cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and instantly stored at −80 ˚C. We stained tissue specimens with eosin and hematoxylin and examined histopathologically. Sections were consisted of >80% tumor cells and were used to extract RNA.

RNA extraction and complementary DNA (cDNA) synthesis. Total RNA was isolated from cancerous tissue and adjacent normal mucosa and was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized from total RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

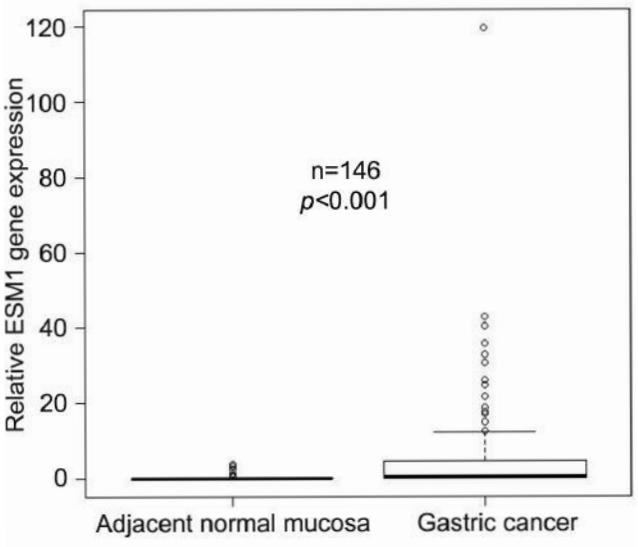

Figure 1. *Comparison of ESM-1 gene expression between gastric cancerous tissues and adjacent normal mucosa.*

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories). PCR reactions were performed in a total volume of 15 μl, which included 0.2 μg of cDNA, 0.4 μM of each primer, 7.5 μl of iQ SYBR Green Supermix containing dATP, dCTP, dGTP and dTTP at concentrations of 400 μM each, and 50 units/ml of iTag DNA polymerase. After the PCR consisted of 3 min at 95 ˚C, the cycling conditions were as follows: 40 cycles of denaturation of the cDNA at 95˚C for 10 s, annealing for 10 s at 60 ˚C for *ESM-1* and β-actin, and a primer extension at 72 ˚C for 20 s, finally hold on 72 ˚C for 10 min. To evaluate the specific mRNA expression in samples, a standard curve was produced for each run, measuring three points of the human control cDNA (Clontech Laboratories, Inc., CA, USA). The concentration of each sample was calculated by relating its crossing point to a standard curve. The PCR primer sequences of *ESM-1* and *β-actin*, as an internal control, are shown in Table I.

Statistical analyses. Gene expression levels were compared between gastric cancer and adjacent normal mucosa by the Wilcoxon's test. The expression of *ESM-1* mRNA was categorized as low or high based on a cut-off value calculated using the maximum chi-square test (χ^2). The optimal cut-off point was selected by the minimum *p*-value method, whereas the internal validity of the cut-off point was evaluated with a two-fold cross-validation approach (21). The relationship between the *ESM-1* mRNA expression and clinicopathological factors were evaluated with the χ^2 test. The survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazards model was used for the univariate and multivariate survival analyses to determine the risk factors. *p*-Values<0.05 were considered to indicate statistical significance. All statistical analyses were performed using the EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for the R software program (The R Foundation for Statistical Computing, Vienna, Austria) (22).

Table II. *Comparison of the clinicopathological factors between high and low ESM1 expression.*

Factors	All patients (n=146)	*ESM1* expression		*p*-Value
		Low (n=63)	High (n=83)	
Age (years), Mean±SD	65.8±9.4	65.2±10.6	66.3±8.4	0.473
Gender				0.721
Male	100 (68.5%)	42 (66.7%)	58 (69.9%)	
Female	46 (31.5%)	21 (33.3%)	25 (30.1%)	
Tumor size (mm)				0.182
<65	69 (47.3%)	34 (54.0%)	48 (57.8%)	
≥65	77 (52.7%)	29 (46.0%)	35 (42.2%)	
Histological type				**0.016**
Differentiated	90 (61.6%)	46 (73.0%)	44 (53.0%)	
Undifferentiated	56 (38.4%)	17 (27.0%)	39 (47.0%)	
Pathological serosal invasion				0.736
Absent	58 (39.7%)	24 (38.1%)	34 (41.0%)	
Present	88 (60.3%)	39 (61.9%)	49 (59.0%)	
Pathological lymph-node metastasis				0.910
Absent	19 (13.0%)	8 (12.7%)	11 (13.3%)	
Present	127 (87.0%)	55 (87.3%)	72 (86.7%)	
Lymphatic invasion				0.430
Absent	34 (23.3%)	17 (27.0%)	17 (20.5%)	
Present	112 (76.7%)	46 (73.0%)	66 (79.5%)	
Venous invasion			0.438	
Absent	36 (24.7%)	18 (28.6%)	18 (21.7%)	
Present	110 (75.3%)	45 (71.4%)	65 (78.3%)	
TNM pathological stage			0.854	
II	42 (28.8%)	19 (30.2%)	23 (27.7%)	
III	104 (71.2%)	44 (69.8%)	60 (72.3%)	

UICC, Union for International Cancer Control; ASA-PS, American Society of Anesthesiologists Physical Status; SD, standard deviation. Bold value shows significance.

Ethics. The present study was conducted in compliance with the 'ethical guidelines for clinical research' and with the Helsinki Declaration of 1975, as revised in 1983. This study was approved by the Institutional Review Board (IRB) of Yokohama City University (approval number: 18-7A-4) and Kanagawa Cancer Center (approval number: epidemiological study-29). Written informed consent for using clinical data without identifying personal information was obtained from all patients the initiation of the study.

Results

ESM-1 mRNA expression. The results revealed that the *ESM-1* mRNA expression was significantly higher in cancer tissue [1.062 (0.000-15.349)] than in normal gastric mucosa [0.426 (0.000-119.618)] ($p<0.001$) (Figure 1).

Patient characteristics. Using the optimal cut-off point of the expression of the *ESM-1* mRNA, patients were classified into those with low expression of *ESM-1* mRNA and high expression of *ESM-1* mRNA. The patients' demographic and clinical characteristics are summarized in Table II. Tumors with an undifferentiated type had a significantly higher *ESM-1* expression than those with a differentiated type ($p=0.016$).

Survival analysis. Patients with a high expression of *ESM-1* mRNA have a significantly worse OS than those with a low expression ($p=0.005$; Figure 2). The 5-year OS rate was 57.5% in the patients with a high expression of *ESM-1* mRNA and 77.8% in those with a low expression of *ESM-1* mRNA. The OS curve in the reference group of pStage II/III gastric cancer patients who did not receive adjuvant S-1 chemotherapy are shown in Figure 3. There was no significant difference in the survival between the patients with a high expression of *ESM-1* mRNA and those with a low expression ($p=0.141$).

The clinicopathological factors were analyzed to determine their prognostic significance for OS (Table III). The univariate analyses demonstrated that the TNM stage and *ESM-1* mRNA expression were significant prognostic factors for OS. The lymph node metastasis was marginally significant prognostic factor. The multivariate analyses demonstrated that the TNM stage and *ESM-1* mRNA expression were significant independent prognostic factors for OS.

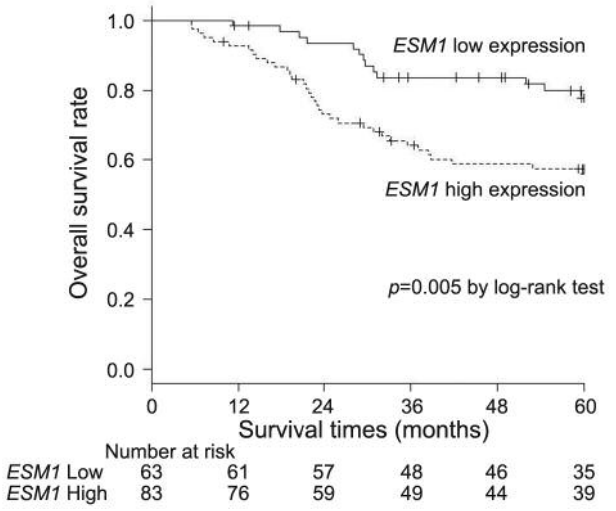

Figure 2. *Comparison of overall survival between high and low ESM-1 gene expression in pathological stage II/III gastric cancer patients who received curative gastrectomy followed by adjuvant S-1 chemotherapy.*

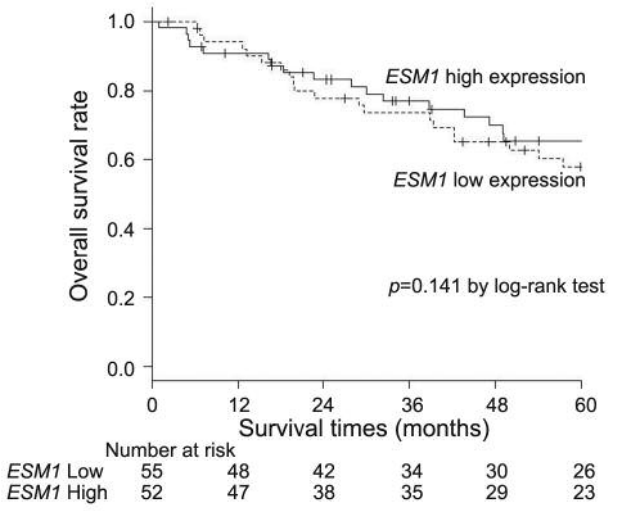

Figure 3. *Comparison of overall survival between high and low ESM-1 gene expression in pathological stage II/III gastric cancer patients who received curative gastrectomy, but did not receive adjuvant S-1 chemotherapy.*

Discussion

The present study evaluated the impact of the *ESM-1* mRNA expression on long-term outcome in pStage II/III gastric cancer patients who received curative resection followed by adjuvant S-1 chemotherapy. The major finding of this study was that the patients with high expression of *ESM-1* mRNA had significantly worse survival than those with low expression of *ESM-1* mRNA. Our results suggested that the *ESM-1* gene expression in cancerous tissue is an important prognosticator in these patients.

We first examined the expression of *ESM-1* mRNA in cancerous tissues and adjacent normal mucosa. Several previous studies have compared the relative mRNA expression of the *ESM-1* gene between various types of cancer tissue and adjacent normal mucosa (10, 20, 23). It was reported that the *ESM-1* expression was higher in gastric cancer tissue than in non-cancerous tissue in 159 samples (20). Our results are consistent with those previous findings, as the expression of *ESM-1* mRNA was significantly higher in the gastric cancer tissue than in the paired adjacent normal mucosa.

We, next, examined the relationship between the *ESM-1* mRNA expression and the clinicopathological factors in gastric cancer. Liu *et al*. reported that the overexpression of *ESM-1* mRNA was significantly correlated with distant metastasis, vascular invasion, and Borrmann type IV after radical resection for gastric cancer (20). Furthermore, they reported that the local expression of ESM-1 was correlated with vascularity and tumor aggressiveness (20). In the present study, tumors with an undifferentiated type had a high *ESM-1* expression.

We then assessed the relationship between *ESM-1* gene expression and long-term outcome in pStage II/III gastric cancer patients who received curative resection followed by adjuvant S-1 chemotherapy. Previous studies reported that a high *ESM-1* mRNA expression was associated with a poor outcome in patients with various cancers, including gastric cancer (17-20). In the present study, a high *ESM-1* mRNA expression was associated with significantly poorer outcomes than a low expression in locally advanced gastric cancer patients who received adjuvant chemotherapy with S-1. Furthermore, a multivariate analysis revealed that a high expression of *ESM-1* mRNA was an independent risk factor for poor outcomes. In contrast, in the patients who did not receive adjuvant S-1 chemotherapy, the overall survival did not significantly differ between the patients with a high expression of *ESM-1* mRNA and those with a low expression of *ESM-1* mRNA. These results suggest that a high expression of *ESM-1* mRNA in gastric cancer tissue indicates a high risk of recurrence in pStage II/III gastric cancer patients who received curative resection followed by adjuvant S-1 chemotherapy. Although further studies are necessary, such patients may be better treated with only close follow-up at an outpatient or with the combination of S-1 plus other anticancer agents.

The mechanism underlying the association of a high expression of *ESM-1* mRNA in cancerous tissue with a poor prognosis in pStage II/III gastric cancer patients who received curative resection followed by adjuvant S-1 chemotherapy is poorly understood at present. However, previous reports have hypothesized several mechanisms:

Table III. *Uni- and multivariate Cox proportional hazards analyses of clinicopathological factors for the overall survival.*

Factors	Number of patients (%)	Univariate			Multivariate		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age (years)				0.320			
<65	58 (50.4%)	1.000					
≥65	57 (49.6%)	0.758	0.439-1.309				
Gender				0.826			
Female	23 (20.0%)	1.000					
Male	93 (80.0%)	1.069	0.593-1.927				
Tumor size (mm)				0.124			
<65	49 (42.6%)	1.000					
≥65	66 (57.4%)	1.551	0.887-2.712				
Histological type				0.980			
Differentiated	17 (14.8%)	1.000					
Undifferentiated	98 (85.2%)	0.993	0.564-1.748				
Pathological serosal invasion				0.219			
Absent	93 (80.9%)	1.000					
Present	22 (19.1%)	1.438	0.805-2.569				
Pathological lymph-node metastasis				0.073			0.769
Absent	59 (51.3%)	1.000			1.000		
Present	56 (48.7%)	2.906	0.906-9.327		1.220	0.324-4.594	
TNM pathological stage				**0.002**			**0.007**
II	58 (50.4%)	1.000			1.000		
III	57 (49.6%)	3.825	1.632-8.965		3.788	1.436-9.993	
Lymphatic invasion				0.357			
Absent	51 (44.3%)	1.000					
Present	64 (55.7%)	1.383	0.694-2.758				
Venous invasion				0.476			
Absent	39 (33.9%)	1.000					
Present	76 (66.1%)	1.274	0.654-2.482				
ESM1 expression				**0.006**			**0.007**
Low	75 (65.2%)	1.000			1.000		
High	40 (34.8%)	2.319	1.270-4.233		2.291	1.250-4.20	

HR, Hazard ratio; CI, confidence interval; UICC, Union for International Cancer Control. Bold values show significance.

ESM-1 is expressed in the tumor endothelium and is upregulated by angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (24). The expression of ESM-1 is also correlated with the balance of positive PKC/NFKB and negative PI3K/AKT/FKHRL1 signaling pathways (25). In addition, it has been recently shown that hypoxia-inducible factor-1a (HIF-1a) was regulated by ESM-1 (26). HIF-1a expression reduced apoptosis in cancer cells through modulation of the expression of apoptotic proteins, such as Bcl-2, Bid, leading to drug resistance against chemotherapeutic agents like 5-fluorouracil (27, 28).

Caution is required when interpreting the current results, since the present study has several potential limitations. First, this study examined the *ESM-1* mRNA expression in cancerous tissues. It will be necessary to examine both the mRNA expression and protein expression using the same specimen to determine the clinical utility of a protein as a biomarker. Second, there is the issue of heterogeneity in cancerous tissue. The sample from which the mRNA was extracted was 5 mm×5 mm×10 μm (for 3 sheets) of cancer tissue. Although that tissue included the deepest part of the tumor, they did not accurately represent the entire tumor.

From the results of this study, it would be suggested to administrate the appropriate regimen based on the *ESM-1* mRNA expression level for pStage II/III gastric cancer in clinical practice. For example, a more efficient adjuvant chemotherapy, such as S-1 plus docetaxel (6), should be administrated if the *ESM-1* mRNA expression level is high in gastric cancer tissue samples after surgery in patients with pStage II/III gastric cancer. On the other hand, no adjuvant chemotherapy and careful monitoring may administrate if the *ESM-1* mRNA expression level is low in patients with stage II gastric cancer who have organ dysfunction, such as in elderly patients. However, validations and prospective studies in are necessary.

In conclusion, a high expression of *ESM-1* mRNA in cancer tissue is an important prognosticator in stage II/III gastric cancer patients who received adjuvant S-1 chemotherapy.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

KK and TO contributed to the conception and design of the study. KK, YK, IH, KH, YM, TA, HF, TY, TO, HC, TY, YM, and TO contributed to the data collection and assembly. All of the authors contributed to the data acquisition and interpretation of the analyzed data. KK wrote the manuscript. All of the Authors contributed to the critical revision of the paper. All of the Authors read and approved the final manuscript.

Acknowledgements

The Authors thank Kazue Yoshihara for her technical support.

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Received October 19, 2019
Revised November 4, 2019
Accepted November 6, 2019